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Dietary Arsenic Affects Dimethylhydrazine-Induced Aberrant Crypt Formation and Hepatic Global DNA Methylation and DNA Methyltransferase Activity in Rats**

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ABSTRACT

Cell culture studies have suggested that arsenic exposure results in decreased *S*-adenosylmethionine (SAM), causing DNA hypomethylation. Previously, we have shown that hepatic SAM is decreased and/or *S*-adenosylhomocysteine increased in arsenic-deprived rats; these rats tended to have hypomethylated DNA. To determine the effect of dietary arsenic on dimethylhydrazine (DMH)-induced aberrant crypt formation in the colon, Fisher 344 weanling male rats were fed diets containing 0, 0.5, or 50 µg As (as $NaAsO_2$)/g. After 12 wk, dietary arsenic affected the number of aberrant crypts ($p<0.02$) and aberrant crypt foci ($p<0.007$) in the colon and the amount of global DNA methylation ($p<0.04$) and activity of DNA methyltransferase (DNMT) ($p<0.003$) in the liver. In each case, there were more aberrant crypts and aberrant crypt foci, a relative DNA hypomethylation, and increased activity of DNMT in the rats fed 50 µg As/g compared to those fed 0.5 µg As/g. The same phenomenon, an increased number of aberrant crypts and aberrant crypt foci, DNA hypomethylation, and increased DNMT tended to hold when comparing rats fed the diet containing no supplemental arsenic compared to rats fed 0.5 µg As/g. The

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data suggest that there is a threshold for As toxicity and that possibly too little dietary As could also be detrimental.

Index Entries: Arsenic; cancer; animal model; dimethylhydrazine; aberrant crypt; methylation.

INTRODUCTION

Through epidemiological studies, arsenic (As) exposure has been associated with lung, skin, bladder, and liver cancer. The US Environmental Protection Agency has classified As as a known human carcinogen (category A). As a result, no threshold exists when determining health effects of As: that is, As is considered toxic at any intake. The resulting dose–response curve for detrimental health effects of As is linear and starts at a concentration of zero arsenic. However, the carcinogenicity of As remains an enigma because, on the one hand, epidemiological studies have associated it with human cancer, but on the other hand, carcinogenesis in rodent models has never been convincingly demonstrated *(1).* Recently, several articles have described alternative interpretations of epidemiological studies linking lower doses of As to cancer. These articles suggest that there are no adverse health effects in the United States from As in drinking water at or below the current maximum contaminant level of 50 µg/L *(2–5).* This would indicate that As toxicity does not follow a linear curve to zero intake. The study by Brown and Ross *(2)* is indicative of a threshold response for As toxicity. Furthermore, Kayajanian *(3)* showed that arsenic exposure (drinking water) in humans shows a "J-shaped" response; that is, compared to exposure to drinking water close to 50 µg/L, low exposure (<50 µg/L) as well as high exposure (>100–150 µg/L) resulted in a higher number of cancers (indicative of a beneficial amount of As).

There is considerable evidence suggesting that As is a beneficial or even essential nutrient for animals and signs of As deprivation have been reported for the chick, goat, minipig, rat, and hamster *(6–9).* The biological responses to As deprivation in laboratory animals suggest an interaction with methionine metabolism. Studies of As deprivation show that rats fed diets containing <10 ng As/g have decreased hepatic concentrations of *S*-adenosylmethionine (SAM) and increased *S*-adenosylhomocysteine (SAH) when compared to rats fed 0.5 µg As/g diet *(10).* We have also observed that hepatic DNA tends to be hypomethylated in As-deprived rats *(10).*

Arsenic, however, is best known for its toxicity and carcinogenicity. Animal and cell culture studies have tested various hypotheses of the mechanism(s) of the carcinogenicity of As. One proposed, much studied mechanism relates to the methylation of As. Methylation of As has long been viewed as a detoxification process because methylated arsenicals are generally less toxic and more readily excreted *(11).* Methylation of As is an enzymatic process requiring SAM-dependent arsenic methyltransferases *(12).* The hypothesis states that methylation of arsenic consumes SAM and,

with depletion of SAM, less As is methylated and, as a result, inorganic arsenic accumulates in the body and causes direct toxic effects. Also, because SAM is consumed, the effects of decreased tissue concentrations of SAM follow (e.g., altered DNA methylation) *(11,13–15).* Although cell culture studies have shown that As exposure decreases the concentration of SAM and/or increases the concentration of SAH, no human or animal study has shown that chronic or acute As exposure decreases tissue SAM. For example, Zhao et al. *(1)* exposed cells (liver cell line developed from Fisher 344 rats) for 18 wk to chronic As and found that the concentration of SAM in As-transformed cells was significantly decreased compared to non-transformed cells. Also, DNA hypomethylation occurred and DNA methyltransferase (DNMT) activity decreased in As-transformed cells. Changes were dose and time related for As exposure. The authors suggested that "taken together, these results strongly point toward hypomethylation of DNA as the causative factor in arsenic-induced malignant transformation."

If SAM is decreased and/or SAH increased as a result of As exposure, inhibition of methyltransferase enzymes might occur. Finkelstein and Martin *(16)* reported that a liver SAM/SAH ratio of about 1.5 is consistent with inhibition of SAM-dependent transmethylation reactions. Another report cited Cantoni, who calculated that when the SAM/SAH ratio drops to 1.6, there is a 20–80% inhibition of maximal activities of transmethylation reactions *(17).* A decrease in SAM/SAH ratio (through a decrease in SAM and/or increase in SAH) and consequent DNA hypomethylation is associated with an increased incidence of cancer *(18–20).*

Thus, because a main proposed mechanism of arsenic's carcinogenicity relates to As decreasing SAM and the fact that As deprivation can result in decreased SAM and increased SAH, we performed an experiment to test the hypothesis that compared to DNA from rats fed 0.5 μg As/g diet, DNA from rats fed deficient or excess As will be hypomethylated. Also, numerous studies have shown that As is not a direct carcinogen but might act as a promoter. Therefore, all rats were injected with dimethylhydrazine (DMH), a cancer-causing agent. Injection of DMH results in the formation of aberrant crypts, a preneoplastic biomarker for colon cancer susceptibility. Thus, the purpose of this study was to evaluate the effect of dietary As on methyl metabolism, aberrant crypt formation, and DNA methylation.

MATERIALS AND METHODS

Animals and Diets

Weanling male Fischer 344 rats (n=60) were purchased from Sasco (Omaha, NE). All rats were housed individually in stainless-steel wire-bottomed cages in a room with controlled temperature and light. Rats were provided free access to demineralized water and purified diet. The basal diet (20% high protein casein, 67% ground corn, 7% soybean oil, and added vitamins and minerals) was reported by Uthus *(21)* but modified to contain

10.5 mg cupric carbonate (55% Cu) and 4 mg folic acid/kg diet. The As content of the diet and whole blood was measured by hydride generation and atomic absorption spectrometric methods *(21,22);* the basal diet contained approx 10 ng As/g diet. The basal diet was supplemented with 0, 0.5, or 50 µg As/g diet as sodium arsenite. After 3 wk of consuming the experimental diets, all rats (n=20/group) were given two interperitoneal injections, separated by 1 wk, of dimethylhydrazine (DMH) (25 mg/kg body weight). Rats consumed the same diets for an additional 8 wk.

This study was approved by the Animal Care Committee of the Grand Forks Human Nutrition Research Center, and the rats were maintained in accordance with the guidelines for the care and use of laboratory animals.

Sample Collection

Food was withheld overnight before rats were anesthetized with xylazine (Rompon; Moboay, Shawnee, KS) and ketamine (Ketaset; Aveco, Fort Dodge, IA) and killed by exsanguination. Blood was collected by cardiac puncture into syringes containing EDTA such that the final concentration was approx 1 g EDTA/L blood. For aberrant crypt analysis (n=20/diet), the colon and rectum were removed, flushed with 9 g/L NaCl, opened longitudinally, and fixed flat between paper towels in 700 mL/L ethanol and stored at 4°C before analysis.

Analysis of Aberrant Crypt Foci

The fixed colon and rectum were stained with 1 g/L methylene blue in 0.1 mol/L sodium phosphate buffer (pH 7.4). Aberrant crypt foci and the total number of aberrant crypts were scored without knowledge of the dietary treatment by using a dissecting microscope to visualize the aberrant crypt foci *(23).*

Genomic DNA Methylation

To assess the methylation status of CpG sites in genomic DNA, the in vitro methyl acceptance capacity of DNA was determined by using [^{3}H-methyl]SAM as a methyl donor and a prokaryotic CpG DNA methyltransferase *(24).* The endogenous DNA methylation status is reciprocally related to the exogenous ^{3}H-methyl incorporation. Briefly, DNA (2 µg) was incubated with 185 kBq of [^{3}H-methyl]SAM (Amersham Life Science, Piscataway, NJ), 4 U of SSS1 methyltransferase (New England Biolabs, Beverly, MA), 1X SSS1 buffer (50 mmol/L NaCl, 10 mmol/L Tris-HCl, 10 mmol/L EDTA, and 1 mmol/L dithiothreitol, pH 8.0) *(24,25).*

DNMT Activity

DNA methyltransferase activity was determined by the procedure of Issa et al. *(26)* as modified by Davis and Uthus *(27).* Liver (10 µg protein) was incubated for 2 h. The results are expressed as Bq per 10 µg cellular protein per 2 h incubation.

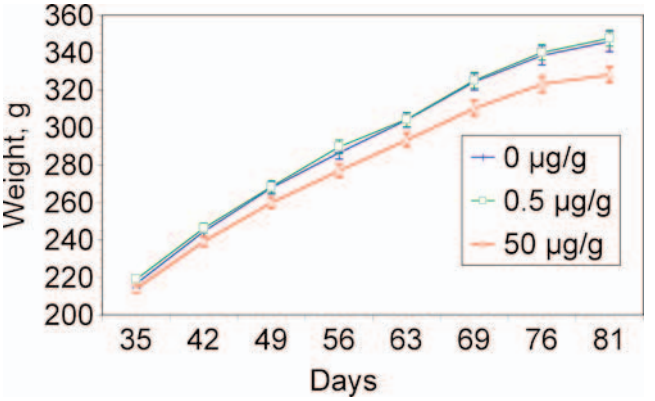

Fig. 1. Rat growth from 35 d until the end of the experiment (81 d). At d 56, 69, 76, and 81, rats fed 50 µg arsenite/g diet weighed significantly less ($p<0.05$) than those fed no arsenic (0 µg/g) or 0.5 µg arsenite/g. Values are means ± SEM; $n = 20$ per group.

Hepatic S-Adenosylmethionine and S-Adenosylhomocysteine

Both SAM and SAH were determined by the procedure of Wagner et al. *(28)* as modified by Davis and Uthus *(27)*.

Plasma Homocysteine and Glutathione

Total homocysteine and total glutathione were determined by the procedure of Durand et al. *(29)*.

Statistical Analysis

The data were analyzed by analysis of variance (ANOVA) using the SAS general linear models program (SAS Version 8.02; SAS Institute, Cary, NC). Tukey's contrasts were used to differentiate among means for variables affected by dietary As. Values are reported as means ± SEM; n=20 per group unless otherwise noted. The aberrant crypt data were analyzed by using a generalized linear model procedure. We assumed that these data followed a Poisson distribution rather than a normal distribution.

RESULTS

After 56 d on the experimental diets, rats fed 50 µg As/g diet weighed significantly less ($p<0.05$) than those fed 0.5 µg As/g diet or no supplemental As (Fig. 1). At the termination of the experiment (81 d), rat weights (g) were 346±5.4 (0 As), 348±4.2 (0.5 As), and 328±4 (50 As).

Liver SAM, SAH, and SAM/SAH were not affected by dietary As (data not shown). Also, plasma total homocysteine and plasma total glutathione were not affected by dietary arsenic (data not shown).

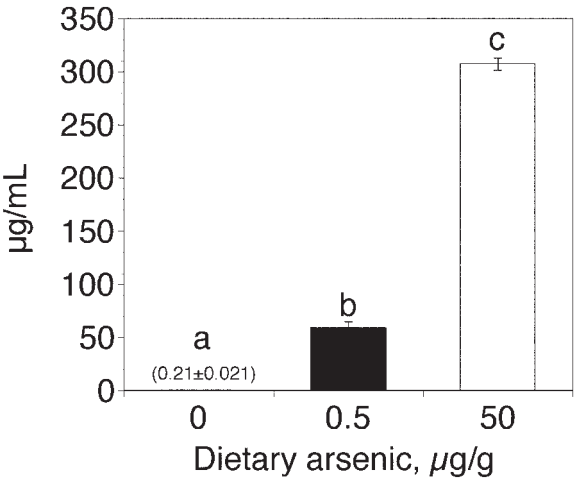

Fig. 2. Whole-blood As concentrations from dimethylhydrazine-treated rats fed 0, 0.5, or 50 µg arsenite/g diet. Arsenic effect; $p<0.0001$. Means without common letters differ; $p<0.05$. Values are means ± SEM; $n = 20$ per group.

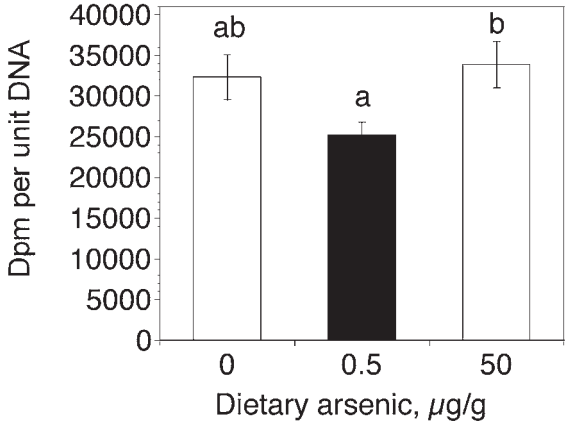

Fig. 3. Hepatic global methylation status from dimethylhydrazine-treated rats fed 0, 0.5, or 50 µg arsenite/g diet. The extent of DNA methylation is inversely proportional to the incorporation (dpm) of ^{3}H-methyl from [^{3}H-methyl]*S*-adenosylmethionine (see Materials and Methods section). Arsenic effect; $p<0.04$. Means without common letters differ; $p<0.05$. Values are means ± SEM; $n = 20$ per group.

Whole-blood As, used as an indicator of arsenic status in the rat, increased significantly with increasing dietary arsenic (Fig. 2).

Dietary arsenic affected liver DNA methylation (Fig. 3). Hepatic DNA from rats fed 50 µg As/g diet showed ($p<0.05$) a relative global hypomethylation compared to DNA from rats fed 0.5 µg As/g diet. DNA

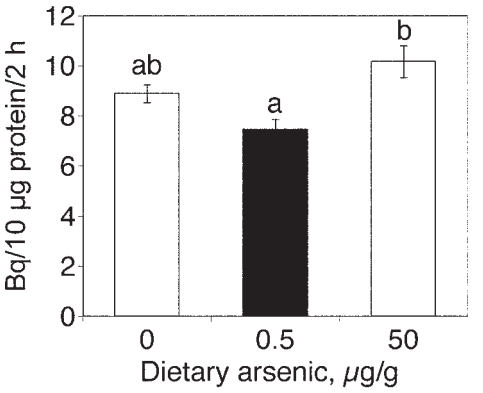

Fig. 4. Specific activity of hepatic DNMT in dimethylhydrazine-treated rats fed 0, 0.5, or 50 µg arsenite/g diet. Arsenic effect; $p<0.003$. Means without common letters differ; $p<0.05$. Values are means ± SEM; $n = 9$ per group.

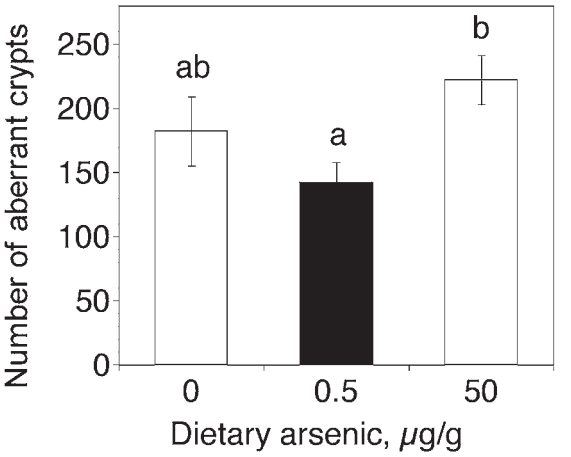

Fig. 5. The number of aberrant crypts in dimethylhydrazine-treated rats fed 0, 0.5, or 50 µg arsenite/g diet. Arsenic effect; $p<0.035$. Means without common letters differ; $p<0.05$. Values are means ± SEM; $n = 20$ per group.

from rats fed no supplemental As tended to be hypomethylated compared to DNA from rats fed 0.5 µg/g diet. Liver DNMT followed this same pattern (Fig. 4). The activity of DNMT was higher ($p<0.05$) in rats fed 50 µg As/g compared to rats fed 0.5 µg As/g diet. DNMT from rats fed no supplemental As tended to be higher than DNMT from rats fed 0.5 µg As/g diet.

The number of aberrant crypts and aberrant crypt foci was increased by high-As feeding compared to rats fed 0.5 µg As/g diet (Figs. 5 and 6). The number of aberrant crypts and aberrant crypt foci tended to be higher in rats fed no supplemental As compared to those fed 0.5 µg As/g diet.

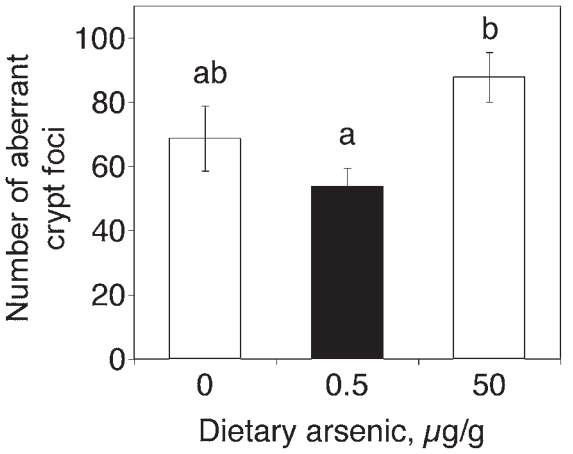

Fig. 6. The number of aberrant crypt foci in dimethylhydrazine-treated rats fed 0, 0.5, or 50 μg arsenite/g diet. Arsenic effect; $p<0.035$. Means without common letters differ; $p<0.05$. Values are means ± SEM; $n = 20$ per group.

DISCUSSION

This study demonstrated that compared to rats fed 0.5 μg As/g diet, rats fed high dietary As (50 μg/g, as inorganic arsenite) had hepatic global DNA hypomethylation, increased specific activity of liver DNMT, and increased number of aberrant crypts and aberrant crypt foci. Compared to rats fed 0.5 μg As/g, rats fed diets devoid in As tended to have DNA hypomethylation, increased DNMT, and increased number of aberrant crypts and foci. Our initial hypothesis was that compared to rats fed 0.5 μg As/g, rats fed diets devoid or high in As would have decreased SAM and/or increased SAH, which would result in inhibition of DNMT, DNA hypomethylation, and increased aberrant crypt formation. DNA global hypomethylation is an early event in colorectal tumorigenesis *(30)*. Others have shown that the degree of hypomethylation associated with colon cancer ranged from about 10% to 30% as measured by 5-methylcytosine content *(31)* or immunostaining *(30)*. Although we found DNA hypomethylation (approximately a 30% change as measured by the methyl acceptance assay) and an increase in number of aberrant crypts, we found no effect on hepatic SAM, SAH, or SAM/SAH ratio (because the colon was fixed and used for determination of aberrant crypts, SAM and SAH status in colon was not assessed) and an increase in liver DNMT activity. This suggests a mechanism other than consumption of methyl groups (i.e., decrease in SAM and/or increase in SAH concentrations) and concomitant inhibition of DNMT.

Previous studies *(8,10)* have shown that As-deprived rats have decreased hepatic SAM and/or increased SAH. We saw no such effect in this experiment, possibly the result of DMH treatment. Halline et al. *(32)* reported that treatment with DHM resulted in increased methionine

adenosyltransferase (MAT) activity and the concentration of SAM, but not SAH, in the distal colon of the rat. In their study, rats were injected with DMH (20 mg/kg body wt) once per week for 5 wk and then killed 1 wk after the last injection. In our study, however, rats were injected with DMH once per week for 2 wk and killed 8 wk after the last injection. Although MAT activity was increased by DMH treatment, Halline et al. *(32)* stated that, based on prior observations in a number of different rat tissues, it was unlikely that this increase accounted for the increased concentration of SAM. Furthermore, in a study with dietary selenium as a variable, we found no effect of DMH treatment (identical DMH treatment and same age, strain, and sex of rat as describe in this publication) on liver or colon SAM, SAH, or SAM/SAH ratio (Uthus and Davis, unpublished findings). Because of our study design, we were able to measure only hepatic SAM or SAH. The liver, however, contains a high Michaelis constant MAT isoenzyme (MAT III) that can respond to variable concentrations of methionine *(33).* Also, SAM is a positive effector of MAT III *(33).* Because of this and because of its central role in metabolism, the liver (as opposed to extrahepatic tissues) usually shows the greatest treatment effect influencing SAM concentrations. Furthermore, the isozyme of MAT found in extrahepatic tissues, MAT II, could be near its maximal capacity at normal tissue concentrations of methionine and it is inhibited by SAM. Consequently, the concentrations of SAM in extrahepatic tissues tend to be relatively constant *(33).* This supports the notion that colon SAM and SAH were not affected by dietary As. Also, not all studies have found a correlation between colon SAM or SAH and colon tumorigenesis. Schmutte et al. *(34)* examined the level of DNMT expression and the concentrations of SAM and SAH in human colon tissues. They found that average DNMT mRNA expression levels were 3.7-fold elevated in tumor tissues compared with surrounding normal mucosa from the same patient. Extracts from the same tissues showed that SAM concentrations were not reduced below the K_m value for the mammalian enzyme and that the ratio of SAM/SAH did not differ significantly.

Because reduction of As to trivalency is necessary for its oxidative methylation, glutathione (GSH) or some other thiol is required *(12,35).* Findings by Csanaky et al. *(11)* suggested that the toxicity of As (as arsenite) is not the result of depletion of SAM but the result of arsenite–sulfhydryl, interactions including depletion of GSH, inhibition of SH enzymes, and depletion of ATP. These studies were done in rats injected with arsenite and represent an acute, rather than chronic, exposure. They actually found an increase in hepatic SAM and SAH as a result of As treatment. We found no effect on hepatic SAM or SAH and no effect on plasma total GSH.

The mechanism resulting in increased aberrant crypts and aberrant crypt foci with feeding high As is not known. Numerous studies indicate that As is not a direct carcinogen but a cocarcinogen. For example, studies by Rossman et al. *(36,37)* are consistent with the hypothesis that As acts as a cocarcinogen with a second (genotoxic) agent and showed that As

inhibits DNA repair (but not via inhibition of DNA repair enzymes) and/or enhances positive growth signaling. Thus, the results of feeding high As on aberrant crypts and aberrant crypt foci seen in this study could be the result of altered DNA repair and DNA hypomethylation, which is associated with altered gene expression.

The mechanism resulting in the tendency for As deprivation to increase aberrant crypts and aberrant crypt foci is also unknown. Previous studies that have not used DMH have shown that As deprivation results in decreased liver SAM and/or increased SAH *(8,10).* Possibly, As deprivation is acting though localized effects on SAM, SAH, or GSH. In a previous experiment *(38),* we found that in female rats fed a diet low in As (<10 ng/g) compared to controls fed a diet containing 0.5 μg As (as arsenite)/g there was a significant decrease in SAM/SAH ratio (mainly the result of increase SAH) and that liver and plasma total GSH tended to be decreased.

Whatever the mechanism(s) is, our results show that both high and very low dietary As result in global DNA hypomethylation, increased activity DNMT, and increased formation of aberrant crypts and aberrant crypt foci. Global DNA hypomethylation with concomitant increased activity or expression of DNMT is commonly found in tumorigenic cells *(39–42).* Increased activity or expression of DNMT with concurrent DNA hypomethylation seems counterintuitive. However, to carry out maintenance methylation, DNMT1 must be recruited to sites of DNA replication by PCNA binding. If this binding is disrupted, as shown by Chuang et al. *(39),* then DNA methylation might be disrupted *(43).*

In conclusion, a number of reports show that abnormal methylation patterns occur early in colorectal cancer *(44)* and that these changes consist mainly of global DNA hypomethylation, regional DNA hypermethylation, and overexpression of DNMT1 *(44).* We show that As deprivation or excess produces similar effects. Although there might be different mechanisms of action, in DMH-exposed rats both As deprivation and As excess resulted in increased aberrant crypts and aberrant crypt foci, preneoplastic biomarkers for colon cancer susceptibility. These data suggest that there is a threshold for As toxicity and that possibly too little dietary As could also be detrimental.

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